Exhibit 10

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Immunology

Tumor rejection antigens of chemically induced sarcomas of inbred mice

(cell surface antigens/M_r 96,000 glycoprotein/tumor-enhancing activity)

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ABSTRACT **Chemically induced sarcomas of inbred mice are immunogenic in syngeneic hosts, and preimmunization with tumor cells leads to resistance to subsequent tumor transplants. The tumor rejection antigens (TRAs) that mediate this reaction are highly specific for each tumor; cross-protection between different syngeneic sarcomas is rare. Isolated membrane and cytosol fractions from two antigenically distinct BALB/c sarcomas, Meth A and CMS5, have TRA activity, and biochemical characterization of the active components from the cytosol and plasma membranes of these two tumors identified a glycoprotein of M_r 96,000. Immunization with unfractionated Meth A cytosol frequently leads to tumor enhancement, but the tumor-enhancing activity (TEA) is lost on fractionation and TRA activity becomes demonstrable. As Meth A and CMS5 lack expression of murine leukemia virus (MuLV) antigens or transcripts, MuLV-related antigens cannot be involved in the TEA or TRA activities of these tumors. In contrast to the lack of cross-reactivity between Meth A and CMS5 TRAs in transplantation tests, rabbit antiserum prepared against the Meth A M_r 96,000 antigen reacted with the CMS5 M_r 96,000 antigen. In view of the biochemical and antigenic similarities of Meth A and CMS5 TRAs, we propose that structurally related but distinct M_r 96,000 glycoproteins are expressed in chemically induced sarcomas and that these molecules are responsible for the individually specific immunogenicity of these tumors.**

The tumor rejection antigens (TRAs) of chemically induced sarcomas of mice have been the object of much interest since their discovery more than two decades ago (1–4). TRAs are demonstrated by their ability to induce resistance to tumor transplants in specifically immunized syngeneic recipients. A striking feature of TRAs is their diversity; each sarcoma appears to have its own unique antigen or set of antigens, because immunization against any one tumor generally offers no protection against any other syngeneic sarcoma. The identity of TRAs and the basis for their extensive polymorphism are unclear. Among the possibilities that have been suggested are (*i*) preexisting antigenic diversity in normal cells (5), (*ii*) reexpression of embryonic or fetal antigens (6–9), (*iii*) antigens encoded by altered or derepressed genes of the major histocompatibility complex (MHC) (10), (*iv*) antigens encoded by genes linked to the immunoglobulin heavy chain (*Igh*) locus (11), (*v*) antigens related to recombinant murine leukemia viruses (MuLV) (12, 13), and (*vi*) epigenetic errors in membrane assembly (14).

To facilitate the isolation and identification of TRAs, considerable effort has gone into developing serological probes to detect these antigens. This has proven to be extremely difficult, and only in the case of BALB/c Meth A sarcoma have antibodies to surface antigens with TRA-related characteristics been repeatedly generated (12, 15, 16). For this reason, attempts to isolate TRAs have relied on transplantation tests to monitor antigen purification, and DuBois *et al.* (17–19) and other workers (20–23) have characterized cellular fractions with TRA activity. In the present report, we describe the purification and characterization of TRAs from two antigenically distinct BALB/c sarcomas and compare their properties with antigens isolated by other investigators.

MATERIALS AND METHODS

Mice and Tumor Lines. Inbred BALB/c mice were obtained from our mouse colonies. BALB/c sarcomas Meth A and CMS5 have been described by DeLeo *et al.* (12).

Preparation of Cytosol and Plasma Membranes. Subcellular fractions were obtained by the method of DuBois *et al.* (19).

Column Chromatography. Concanavalin A (Con A)-Sepharose, DEAE-Sepharose CL-6B, and FPLC Mono Q columns were obtained from Pharmacia. Immunoaffinity chromatography was performed by the method of Livingston (24). For details, see Fig. 2 legend.

NaDodSO$_4$/PAGE. NaDodSO$_4$/PAGE was performed according to Laemmli (25) and the gels were stained with silver by the method of Oakley *et al.* (26).

Protein Electrophoretic Blotting. Protein blotting was carried out by the method of Towbin *et al.* (27).

Cell Surface Labeling. Cells were surface labeled by lactoperoxidase-mediated iodination (28).

RESULTS

Tumor Rejection Assays. Two transplantable sarcomas of BALB/c origin, Meth A ascites and CMS5, were chosen for these studies because of their well-defined immunogenicity, lack of cross-protective antigens, and absence of MuLV-related antigens. Our standard protocol involves immunizing mice by two injections one week apart followed by a tumor challenge 1 week after the second injection. For immunization, mice are injected subcutaneously in the dorsal cervical region, and for challenge, the injection is given intradermally in the shaved dorsal flank. The following points have been considered in developing this protocol: (*i*) Each mouse is immunized with antigen extracted from a constant number of tumor cells or weight of tumor tissue rather than with a constant amount of protein. On the basis of past experience, we define one unit of antigen as the amount derived from 25 $\times$ 10^6 Meth A ascites cells or from 1 g (wet weight) of CMS5. (*ii*) In order to avoid the possibility of tumor rejection due to reactions against the calf serum components used in culturing tumor cells, antigen for immunization is extracted from tumors grown *in vivo*. The tumor cells used for challenge are

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Abbreviations: Con A, concanavalin A; MuLV, murine leukemia virus; TEA, tumor-enhancing activity; TRA, tumor rejection antigen.

grown *in vivo* in the case of Meth A ascites, while tissue cultured cells are used for CMS5 challenge. Thus, the possibility that tumor rejection is due to reactions against heterologous serum components is bypassed in studies with Meth A and is minimized in studies with CMS5. (*iii*) The number of tumor cells selected for challenging immunized mice has been arrived at in the following way: mice immunized with 2 units of antigen (in the form of irradiated cells or tumor fragments) were challenged with different numbers of tumor cells, and the highest tumor challenge rejected by immunized recipients has been used as the challenge level for monitoring TRA activity. For Meth A, the challenge dose was 125,000 cells and for CMS5, 75,000 cells. The tumor rejection assays shown in the figures represent individual experiments; each experiment was repeated at least two to four times.

Purification and Identification of Meth A TRA. Meth A ascites cells were harvested and cytosol and plasma membrane preparations were derived. The plasma membrane preparation was further extracted with 0.1% sodium deoxycholate, and a detergent-insoluble matrix and a soluble fraction were obtained. The detergent-solubilized fraction was extensively dialyzed to obtain a water-soluble fraction of the plasma membrane. Mice were immunized with the cytosol or the water-soluble membrane fraction and were challenged with Meth A cells. Tumor growth was monitored on a daily basis and was recorded at 7- to 10-day intervals. The plasma membrane fraction elicited a clear tumor rejection response, whereas mice immunized with cytosol showed no inhibition of tumor growth and, in fact, frequently showed enhanced tumor growth (Fig. 1). However, fractionation of the cytosol on Con A-Sepharose 4B or DEAE-Sepharose CL-6B revealed significant TRA activity in the Con A-bound fraction and the DEAE-bound fraction (Fig. 2). This unmasking of TRA activity in Meth A cytosol suggests that the unfractionated cytosol has a factor(s) that neutralizes the immunogenicity of Meth A TRA; we refer to this as tumor enhancing activity (TEA) because tumor enhancement is often seen after immunization with whole Meth A cytosol (see *Discussion*).

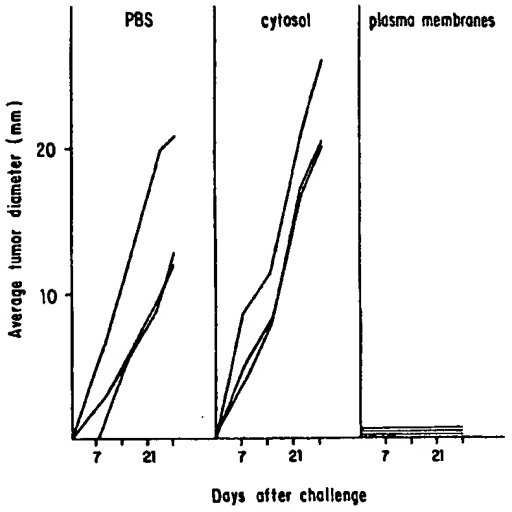

FIG. 1. Growth of Meth A in BALB/c female mice after immunization with Meth A cytosol (2 units per mouse) or Meth A membranes (2 units per mouse). Unimmunized controls were injected with Dulbecco's phosphate-buffered saline (PBS). Tumor challenge: 125,000 Meth A cells. Each line represents tumor growth in a single mouse.

To purify the TRA from Meth A cytosol, the Con A-bound fraction was applied to a DEAE-Sepharose CL-6B column and fractions were eluted with a 0–1 M NaCl gradient. The TRA activity eluted as a broad peak between 300 and 400 mM NaCl. This peak was applied to a Mono Q FPLC column and the resulting fractions were tested for TRA activity (Fig. 2). Though more than one fraction showed TRA activity, the major activity eluted at 400 mM NaCl. This preparation was analyzed by $NaDodSO_4$/PAGE and showed a prominent protein band of M_r 96,000 and a minor species of M_r 150,000 under both reducing and nonreducing conditions (Fig. 3). The two bands were individually excised from a preparative gel and the proteins were eluted, dialyzed against PBS, and tested for TRA activity. The M_r 96,000 protein but not the M_r 150,000 protein elicited tumor rejection. Approximately 2.5 μg of purified M_r 96,000 protein from Meth A cytosol correspond to one unit of Meth A TRA. We have noticed that purified M_r 96,000 antigen tends to break down into discrete components of M_r 94,000 to 65,000 during storage at 4°C.

TRA has also been purified from the soluble fraction of Meth A plasma membranes. This fraction was applied to a Con A-Sepharose column, and the bound and unbound fractions were tested for TRA activity. Although a small proportion of the activity bound to the column, most of the activity appeared in the unbound fraction. The Con A-unbound material was then applied to a DEAE-Sepharose CL-6B column, from which TRA activity eluted at 300 mM NaCl. This fraction was applied to a Mono Q FPLC column, and TRA activity eluted at 300 mM NaCl. $NaDodSO_4$/PAGE analysis of this fraction showed a major protein band of M_r 96,000.

Purification and Identification of CMS5 TRA. To determine whether TRAs of other tumors would have the characteristics of Meth A TRA, we purified TRA from the antigenically distinct sarcoma CMS5. Cytosol and plasma membrane fractions of CMS5 were tested in tumor rejection assays. Plasma membrane preparations reproducibly showed TRA activity, but the level of activity in individual cytosol preparations varied from undetectable to significant. However, irrespective of whether or not unfractionated cytosol showed TRA activity, fractionation on a Con A-Sepharose column consistently revealed TRA activity in the Con A-bound fraction and, to a more variable degree, in the Con A-unbound fraction. Con A-bound fractions were applied to a DEAE-Sepharose CL-6B column, and fractions eluting at 200–300 mM NaCl were observed to have TRA activity. This material was applied to a Mono Q FPLC column, and the fractions eluting between 200 and 320 mM NaCl showed TRA activity. All fractions from the Mono Q column were analyzed by $NaDodSO_4$/PAGE, and a prominent band of M_r 96,000 was observed in fractions eluting between 200 and 320 mM NaCl but not in other fractions (Fig. 3). Only fractions containing the M_r 96,000 band elicited resistance to CMS5 in tumor rejection assays, and purified CMS5 M_r 96,000 antigen containing no other bands had TRA activity.

Specificity of Meth A and CMS5 TRAs. TRA was purified from the cytosol of Meth A and CMS5 by sequential Con A-Sepharose and DEAE-Sepharose chromatography. As shown in Fig. 4, mice immunized with Meth A TRA resisted challenge with Meth A but not CMS5; conversely, mice immunized with CMS5 TRA resisted challenge with CMS5 but not Meth A. Thus, tumor immunity elicited by isolated Meth A and CMS5 TRAs showed the same specificity as immunity elicited by intact tumor cells.

Generation and Characterization of a Rabbit Antiserum Against Meth A TRA. A rabbit antiserum against the M_r 96,000 Meth A TRA was generated by repeated injections, 20 μg each, of purified material obtained from preparative

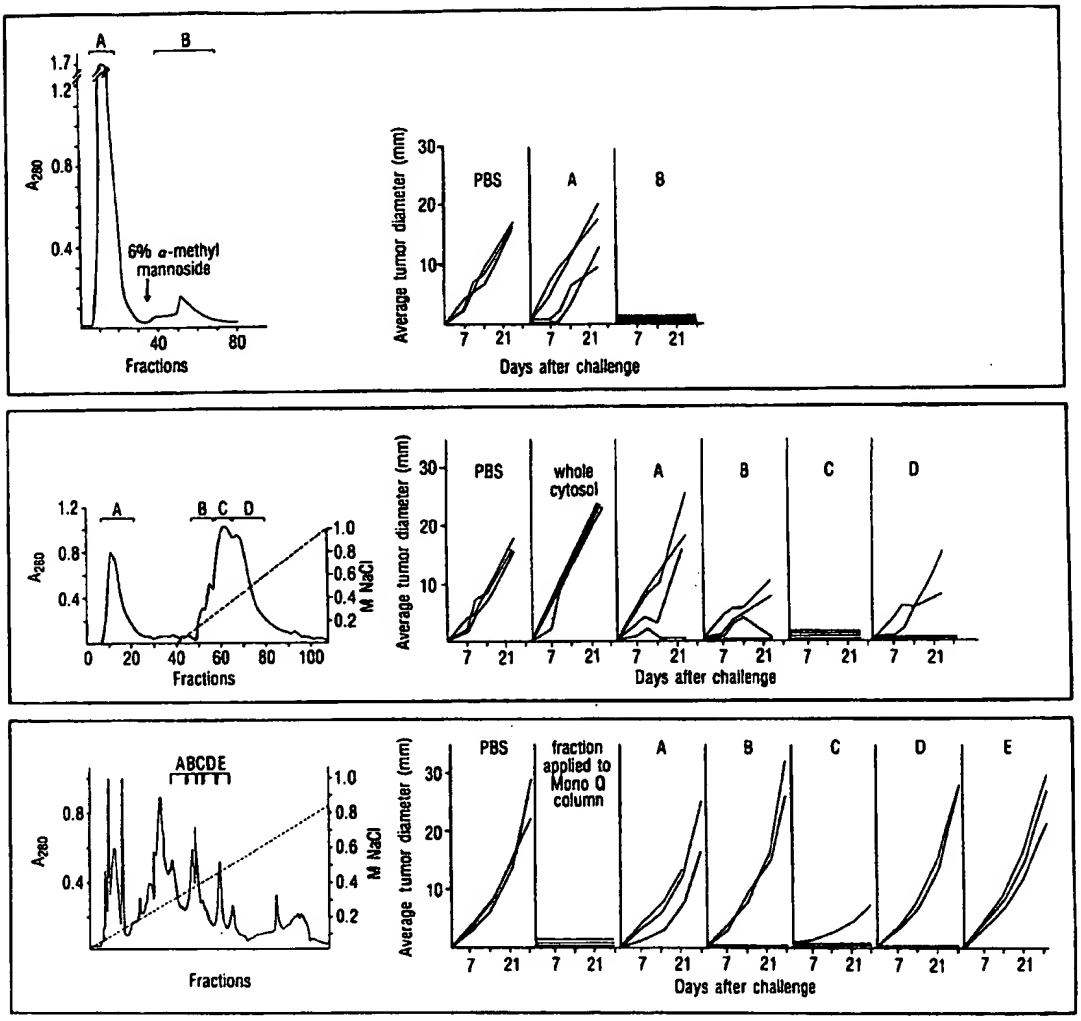

FIG. 2. Purification of TRA from Meth A cytosol by chromatography on Con A-Sepharose (*Top*), DEAE-Sepharose (*Middle*), and Mono Q FPLC (*Bottom*). Con A-Sepharose columns were equilibrated with phosphate-buffered saline containing Ca^{2+} and Mg^{2+} and bound proteins were eluted with 6% methyl α-mannoside in the same buffer. DEAE-Sepharose columns were equilibrated with 20 mM sodium phosphate buffer, pH 7.0, and Mono Q FPLC columns, with 10 mM sodium phosphate buffer, pH 7.0. Bound proteins were eluted with a linear 0.0–1.0 M NaCl gradient. Chromatographic profiles are shown on the left and tumor rejection assays on the right of each panel. Lines represent tumor growth in mice challenged with 125,000 Meth A cells. Fractions (designated by letters) were tested in groups of three or four mice each; each mouse was injected with 2 units of antigen. Unfractionated Meth A cytosol was applied to Con A-Sepharose and DEAE-Sepharose columns. The fraction applied to the Mono Q column was derived from sequential chromatography on Con A-Sepharose (fraction B) followed by DEAE-Sepharose (fraction C).

$NaDodSO_4$/PAGE under nonreducing conditions. Antiserum obtained after four injections detected the M_r 96,000 component in blots of Meth A cytosol and whole membranes and immunoprecipitated a M_r 96,000 molecule from surface-iodinated Meth A ascites cells (Fig. 3) and from Meth A (solid) tumor cells, the original Meth A line from which Meth A ascites was derived. The antiserum did not immunoprecipitate any components from (*i*) surface-labeled CMS5 or CMS13 (another methylcholanthrene-induced BALB/c sarcoma), (*ii*) BALB/c leukemias RVC and RVD and C57BL leukemias RV1 and RV2 induced by radiation leukemia virus, or (*iii*) BALB/c splenocytes, thymocytes, or cultured lung fibroblasts. In complement-mediated cytotoxicity tests with rabbit complement, the antiserum lysed Meth A ascites cells as well as Meth A (solid) tumor cells (titer 1:200) but did not lyse CMS5 or CMS13 cells. Specificity was also analyzed by using protein blots of partially purified TRAs from Meth A and CMS5. The antiserum detected a M_r 96,000 protein in Meth A cytosol and membrane fractions; no proteins were detected in CMS5 cytosol and membranes. However, antiserum obtained after seven injections with Meth A TRA recognized a M_r 96,000 molecule in cytosol and membrane preparations from CMS5 (Fig. 3).

An immunoaffinity column was prepared by covalently linking the IgG fraction of the rabbit antiserum to activated Sepharose. A preparation of Meth A TRA was applied to the column and unbound and eluted fractions were analyzed for TRA activity. The eluted fraction was active in the Meth A tumor rejection assay, whereas the unbound fraction was depleted of TRA activity. $NaDodSO_4$/PAGE of the eluted

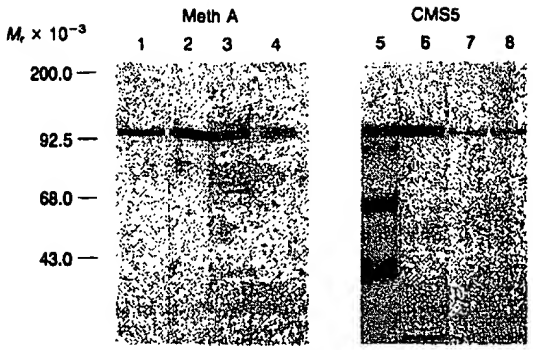

FIG. 3. Characterization of Meth A and CMS5 cytosol and membrane preparations. Lane 1, $NaDodSO_4$/PAGE (nonreducing conditions) and silver staining of fraction derived from Meth A cytosol after sequential chromatography on Con A-Sepharose (fraction B), DEAE-Sepharose (fraction C), and Mono Q (fraction C). This fraction is highly enriched for Meth A TRA. Lane 2, blot of unfractionated Meth A cytosol with rabbit anti-Meth A M_r 96,000 serum (rabbit anti-Meth A TRA). Lane 3, blot of whole Meth A membranes with rabbit anti-Meth A TRA. Lane 4, $NaDodSO_4$/PAGE (nonreducing conditions) of immunoprecipitate of ^{125}I surface-labeled Meth A cells with rabbit anti-Meth A TRA. Lane 5, $NaDodSO_4$/PAGE (nonreducing conditions) and silver staining of fraction derived from CMS5 cytosol after sequential chromatography on Con A-Sepharose, DEAE-Sepharose, and Mono Q FPLC columns. This fraction is highly enriched for CMS5 TRA. Lane 6, blot of CMS5 cytosol fraction shown in lane 5 with rabbit anti-Meth A TRA (after seven injections). Lane 7, blot of unfractionated CMS5 cytosol with rabbit anti-Meth A TRA (after seven injections). Lane 8, blot of whole CMS5 membranes with rabbit anti-Meth A TRA (seven injections). The molecular weight markers are myosin (200,000), phosphorylase B (92,500), bovine serum albumin (68,000), and ovalbumin (43,000).

fraction showed a single band of M_r 67,000; this molecule reacted strongly with the rabbit anti-Meth A M_r 96,000 serum in protein blots. It seems likely that the pH 11.5 buffer used to elute the bound fraction is responsible for generating the M_r 67,000 fragment from the M_r 96,000 component.

Dose Restriction of TRA Activity. To investigate the relation between antigen dose and tumor immunity, mice were immunized with the Con A-bound fraction of Meth A cytosol in doses ranging from 1 unit to 50 units per mouse and were challenged with the 125,000 Meth A cells. The results indicate that administration of TRA causes tumor immunity only within a restricted dose range. Administration of 10 units had virtually no effect on tumor growth, while administration of 20 units caused tumor enhancement (Fig. 5). Mice injected with 50 units died within 12–24 hr; trivial explanations for this toxicity (e.g., residual buffer, $NaDodSO_4$, or high protein concentrations) have been ruled out.

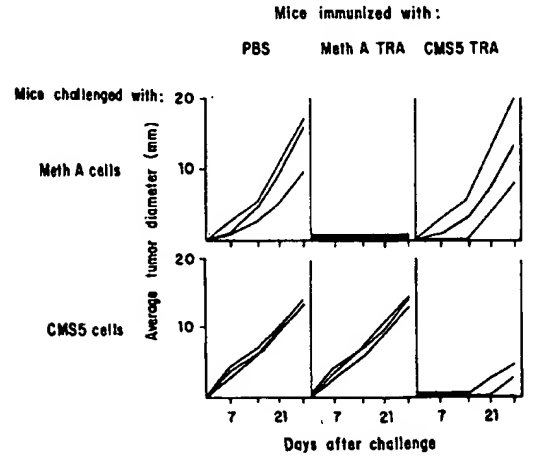

FIG. 4. Growth of Meth A and CMS5 in BALB/c female mice after immunization with Meth A TRA (2 units per mouse) or CMS5 TRA (2 units per mouse). Lines represent tumor growth in mice challenged with 125,000 Meth A cells or 75,000 CMS5 cells.

DISCUSSION

The M_r 96,000 components isolated from Meth A and CMS5 display the immunogenicity and specificity expected of TRAs of chemically induced sarcomas—i.e., they confer immunity against substantial tumor challenges comparable to immunity elicited by trocar pieces and they do not protect against challenges with an independently induced syngeneic sarcoma. DuBois *et al.* (19) have also used Meth A as a source of TRA and have concluded that a M_r 75,000 component represents the active antigen. Table 1 summarizes the characteristics of the M_r 75,000 antigen (subsequently revised to M_r 82,000) described by DuBois *et al.* (19) and the M_r 96,000 antigen described in this report. By a number of criteria, the two antigens are clearly distinguishable. In addition, we have assayed for the presence of the M_r 75,000/82,000 during our fractionation procedures by using an antiserum provided by E. Appella (National Institutes of Health). Fractions enriched in the M_r 75,000/82,000 antigen did not show TRA activity in our tumor rejection tests, and no M_r 75,000/82,000 protein could be detected in purified M_r 96,000 preparations that are highly active in TRA assays. The M_r 75,000/82,000 antigen could be a processed or degraded form of the M_r 96,000 molecule, although the distinct serological and biochemical properties of the two molecules appear to rule out this possibility. DuBois *et al.* (29) have also detected a M_r 86,000 component in a simian virus 40-induced sarcoma (BALB/c mKSA) that has TRA activity against mKSA but not other tumors. A component with similar molecular mass has been isolated from Meth A cytosol, and this M_r 86,000 component elicits transplantation immunity against Meth A but not mKSA or other tumors (30). The M_r 86,000 antigen can be distinguished from the M_r 96,000 antigen identified by us by molecular mass and by failure of a rabbit antiserum to M_r 86,000 (provided by E. Appella) to detect the M_r 96,000 component in electrophoretic blots.

Activation of MuLV is a frequent accompaniment of tumorigenesis in mice, and MuLV structural components are commonly expressed on the cell surface of chemically induced sarcomas (12, 31). Hellstrom *et al.* (32) and Zbar *et al.* (33) have shown that MuLV-related antigens can function as transplantation antigens in syngeneic hosts, but these antigens give rise to cross-reactive protection rather than the

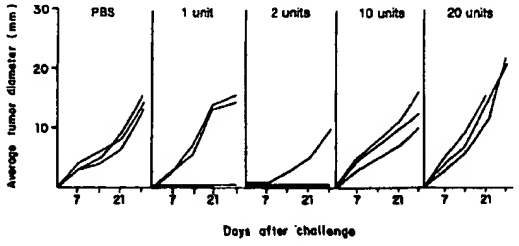

FIG. 5. Growth of Meth A in BALB/c female mice after immunization with 0, 1, 2, 10, or 20 units of Meth A TRA per mouse. Lines represent tumor growth in mice challenged with 125,000 Meth A cells.

Table 1. TRAs of BALB/c sarcoma Meth A: Comparison of the M_r 75,000/82,000 antigen described by DuBois *et al.* (19) and the M_r 96,000 antigen described in the present report

Criterion	M_r 75,000/82,000 antigen	M_r 96,000 antigen
Lectin affinity	No binding to Con A or lentil lectin	Binds to Con A, lentil lectin, and wheat germ lectin
Charge	No (or minimal) binding to DEAE-Sepharose in 20 mM sodium phosphate, pH 7.0.	Binds strongly to DEAE-Sepharose in 20 mM sodium phosphate, pH 7.0; elutes between 300 and 400 mM NaCl
Reactivity with rabbit anti-M_r 75,000/82,000 serum	Reacts in immunoprecipitation and protein blots	No reactivity
Reactivity with rabbit anti-M_r 96,000 serum	No reactivity	Reacts in immunoprecipitation and protein blots

individually specific pattern of protection characteristic of TRAs. DeLeo *et al.* (12) raised the possibility that the extensive polymorphism of gp70 envelope components of recombinant MuLV could be one of the sources of the extensive antigenic variation in chemically induced sarcomas. Lennox (13) has pursued this line of thought and has concluded that the TRAs of chemically induced tumors are polymorphic MuLV-related antigens. This cannot be the case in our studies, since careful scrutiny with broadly reacting serological and molecular probes failed to reveal MuLV-related antigens or transcripts in the two sarcomas analyzed in this report.

Although unfractionated Meth A cytosol has been reported to elicit resistance to transplants of Meth A (18), this was not observed under the conditions of our tests. Rather, we found that unfractionated Meth A cytosol frequently caused enhanced tumor growth. In contrast to the restricted protective effect of Meth A TRA, the tumor-enhancing activity (TEA) of Meth A cytosol was evident in mice when challenged with other methylcholanthrene-induced sarcomas. Unfractionated cytosol from CMS5 lacked detectable TEA, as did cytosol from normal tissues of BALB/c and C57BL mice. However, the consistent recovery of TRA activity from CMS5 cytosol after fractionation on Con A-Sepharose (even when TRA activity could not be detected in unfractionated cytosol) suggests that TEA is present in CMS5, but at lower concentrations than in Meth A and that Meth A and CMS5 TEA have TRA-neutralizing activity. A number of questions about TEA, including the relation between TEA and TRA, and the basis of tumor enhancement by TEA as compared to enhancement by large doses of TRA, await characterization of the factors responsible for TEA.

Note Added in Proof. Amino-terminal sequencing of the M_r 96,000 components from Meth A and CMS5 shows an identical stretch of 14 amino acids.

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